AD_____

Award Number: DAMD17-01-1-0256

TITLE: Structure-Based Approach for Discovery of Small Molecules Inhibitors Targeted at Bcl-2

PRINCIPAL INVESTIGATOR: Shaomeng Wang, Ph.D.

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Annual (1 Sep 2003 - 31 Aug 2004)

4. TITLE AND SUBTITLE
Structure-Based Approach for Discovery of Small Molecules Inhibitors Targeted at Bcl-2

5. FUNDING NUMBERS
DAMD17-01-1-0256

6. AUTHOR(S)
Shaomeng Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Michigan
Ann Arbor, Michigan 48109-1274

E-Mail: shaomeng@umich.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Overexpression of Bcl-2 has been observed in 70% of breast carcinomas and the expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. In this IDEA grant, we propose an effective structure-based approach to discover small molecule inhibitors of Bcl-2 through structure-based 3D-database search over large chemical databases of synthetic compounds or natural products. Using this powerful approach, we have discovered 10 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2. Our studies showed that the most promising small-molecule inhibitors of Bcl-2 we have discovered potently bind to Bcl-2 protein, inhibit cell growth and induce apoptosis in breast cancer cells with high levels of Bcl-2 proteins and display good selectivity in normal cells with low levels of Bcl-2 proteins. One of the most potent Bcl-2 inhibitors achieves a significant anti-tumor activity *in vivo* and represents a highly promising new anti-cancer agent for further evaluation.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction: Bcl-2 is the founding member of the Bcl-2 family proteins and potently inhibits apoptosis in cells. As a potent anti-apoptotic molecule, Bcl-2 contributes to cancer cell progression by preventing normal cell turnover caused by physiological cell death mechanisms. Overexpression of Bcl-2 has been observed in 70% of breast carcinomas. The expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. The experimental three-dimensional (3D) structure of Bcl-2 showed that Bcl-2 has a surface binding pocket into which pro-apoptotic proteins such as Bid, Bim and Bad bind. This pocket is essential for the anti-apoptotic function of Bcl-2 since mutations at this site abolished Bcl-2 biological function. Therefore, we hypothesize that non-peptide, drug-like, cell permeable small molecules that bind to this surface pocket of Bcl-2 will block the anti-apoptotic function of Bcl-2 and may restore the normal apoptotic process in cancer cells with Bcl-2 protein overexpression and make these cancer cells more susceptible to conventional chemotherapy or radiation therapy. Designing of small molecule inhibitors targeting Bcl-2 at this crucial binding site represents an attractive approach for the development of a novel therapy for the treatment of breast cancer with Bcl-2 protein overexpression.

In this IDEA grant, we propose an effective structure-based approach to discover small molecules that bind to the Bcl-2 binding pocket. Specifically, we propose to perform structure-based 3D-database search over large chemical databases containing >500,000 structurally diverse, non-peptide, drug-like synthetic compounds or natural products to identify small molecule candidates that can effectively interact with the Bcl-2 binding pocket. Most promising candidate molecules are then tested in appropriate binding and cellular assays to confirm their activity, specificity and mechanism. For the best Bcl-2 inhibitors identified from this project, they will be further evaluated for their anti-cancer activity *in vivo* and their therapeutic potential for the treatment of human breast cancer with high levels of Bcl-2 protein.

Discovery of novel, non-peptidic, cell permeable Bcl-2 small molecule inhibitors represents the first but very exciting step toward the development of a novel cancer therapy targeted at Bcl-2. The success of this project will pave the way for the development of a small molecule drug through modulation of the Bcl-2 function for the treatment of breast and many other forms of cancers with Bcl-2 overexpression, either alone or in combination with conventional chemotherapeutic drugs or radiation therapy.

Body of the report:

Task 1. Molecular modeling, structure-based database searching, and computational docking (**1-30 months**).

Task 1.1. Extensive molecular dynamics simulation of Bcl-2 through molecular dynamics simulations.

This task was completed and the report was submitted last year.

Task 1.2. Structure-based 3D-database searching on four 3D-databases containing more than 650,000 small organic compounds and natural products to identify most promising small molecule inhibitors that effectively interact with the Bcl-2 surface-binding pocket. (**1-30 months**).

During the past 12 months, we have completed the 3D-database searching of the National Cancer Institute's 3D-database of more than 250,000 synthetic organic compounds and natural products. In addition, we have also performed computational structure-based 3D database searching of the Available Chemical Database (ACD), which contained additional 250,000 synthetic organic compounds and natural products. We have obtained additional 200 promising candidate compounds based upon their predicted binding scores.

Task 2. *In vitro* biological confirmation of potential Bcl-2 inhibitors and mechanism investigations (**3-30 months**).

Task 2.1. Testing of potential small molecule inhibitors of Bcl-2 using an *in vitro* fluorescence polarization (FP) based binding assay.

We have further optimized our sensitive and quantitative *in vitro* fluorescence polarization (FP) based binding assay to test the binding of these potential small molecule inhibitors to Bcl-2 (Enyedy, I. J.; Ling, Y.; Nacro, K.; Tomita, Y.; Wu, X.; Cao, Y.; Guo, R.; Li, B.; Zhu, X.; Huang, Y.; Long, Y.-Q.; Roller, P. P.; Yang, D.; Wang, S.; Discovery of Small-Molecule Inhibitors of Bcl-2 through Structure-Based Computer Screening, *J. Med. Chem.;* **2001**; *44*(25); 4313-4324.) In our

previous assay conditions, we have used a fluorescent peptide tracer, in which a peptide derived from the BH3 domain of the BAK protein was tagged with a fluorescence probe (Flu-Bak-BH3). Although this Flu-Bak-BH3 was adequate for screening these potential small-molecule inhibitors of Bcl-2 identified from our computational 3D database searching, it has a relatively low affinity, which makes it inadequate for determination of potent small-molecule inhibitors of Bcl-2 with high-affinities. To overcome this limitation, we have synthesized a new probe, in which a 21-residue peptide derived from the BH3 domain of another pro-apoptotic protein BID was tagged with a fluorescence tag. This new probe has a K_d value of 10 nM to Bcl-2 and is suitable for accurate determination of the binding affinities of potent small-molecule inhibitors of Bcl-2.

Figure 1. Competitive binding curves of gossypol, (-)-gossypol and (+)-gossypol to Bcl-2 protein, as determined using our fluorescence polarization based binding assay.

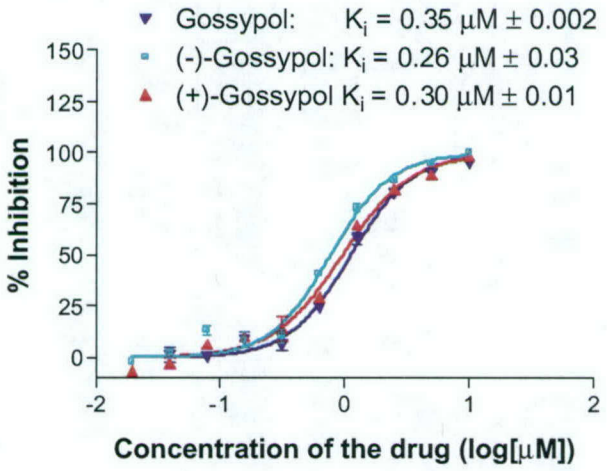
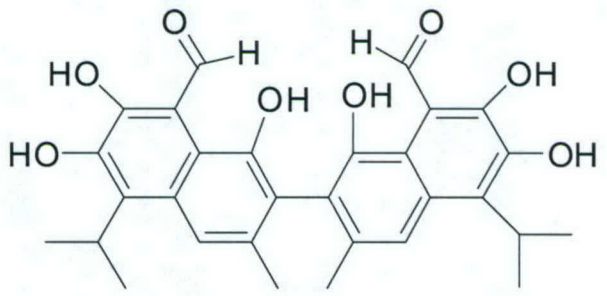


Figure 2. Chemical structure of gossypol.



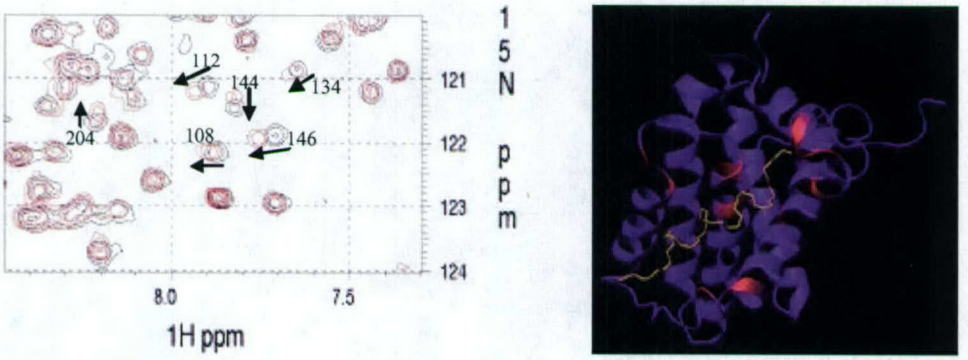
Using this new competitive FP-based binding assay, we have determined the binding affinities for over 100 additional potential small-molecule inhibitors identified from our 3D-database searching. Our work led to the discovery of several classes of new small-molecule inhibitors of

Bcl-2. Among them, Gossypol was one of the most potent small-molecule inhibitors of Bcl-2 we have discovered to date using our computational structure-based database searching strategy. As can be seen from **Figure 1**, gossypol has a K_i value of 350 nM. Using a similar We further determined that gossypol binds to Bcl-xL with a K_i value of 600 nM. Bcl-xL is a closely related homolog of Bcl-2 protein and has been found to be highly overexpressed in human breast cancer cells and tissue. The chemical structure of gossypol is shown in **Figure 2**.

Gossypol is a natural product, isolated from cotton seeds and roots. In the earlier 1980s, gossypol was found to have a potent anti-tumor activity. Although its molecular mechanism of action was not well understood, gossypol was shown to have a novel molecular mechanism different from known anti-cancer drugs. Our discovery that gossypol potently binds to Bcl-2 suggests that the anti-tumor activity of gossypol may be mediated by its binding to and inhibition of Bcl-2 protein in cancer cells.

Gossypol has two enantiomers, i.e. (-)-gossypol and (+)-gossypol. We have therefore synthesized these two enantiomers to evaluate the binding affinities of these two enantiomers to Bcl-2. Interestingly, it was determined that these two enantiomers have similar affinities to Bcl-2 (**Figure 2**).

Figure 3. Probing the interactions between gossypol and the Bcl-xL protein using nuclear magnetic resonance (NMR) method.



The FP binding assay showed that Gossypol and its enantiomers abrogate the interaction between BH3 peptides and both proteins, Bcl-2 and Bcl-xL, but does not provide direct information on where they bind. To conclusively confirm that gossypol and its enantiomers bind to the surface pocket of Bcl-2 and of Bcl-xL where the BH3 domain of pro-apoptotic proteins binds, we performed an analysis using nuclear magnetic resonance (NMR) methods. We chose to use Bcl-

xL for our NMR experiments because Bcl-xL behaves much better in solution. Furthermore, Bcl-2 and Bcl-xL are closely related homologous proteins and have very similar 3D structures. Furthermore, gossypol binds to Bcl-2 and Bcl-xL with similar affinities. Hence, it is likely that gossypol binds to Bcl-2 and Bcl-xL in very similar binding modes.

We employed the heteronuclear single-quantum correlation (HSQC) method with ^{15}N-labeled BCL-XL and obtained the HSQC spectra with Bcl-xL protein alone, with gossypol. We analyzed changes in Bcl-xL structure after the addition of gossypol. Mapping of the observed changes in the NMR spectra upon addition of gossypol into the known three-dimensional structure of Bcl-xL reveals that gossypol mostly affects those residues around the BH3 binding pocket in Bcl-xL (**Figure 3A** and **3B**). As can be seen, the binding of gossypol caused the peak shifts of following residues A108, L112, L134, I144, A146, A204. Most of these residues are indeed around the BH3 binding pocket of Bcl-xL.

Hence, our NMR studies provide a conclusive evidence that gossypol binds to the BH3 binding site, where the pro-apoptotic protein such as BID, BIM and BAD bind.

Task 2.2. Testing the activity of small molecule inhibitors of Bcl-2 in human breast cancer cells.

Despite their similar binding affinities of these two enantiomers to Bcl-2, it was found that (-)-gossypol has a much better activity in inhibition of cell growth, due to, at least in part the larger influence of serum protein to the activity of (+)-gossypol. As can be seen from **Figure 4**, (-)-gossypol is approximately 2-times more potent than racemic gossypol but is 5-10 times more potent than (+)-gossypol in inhibition of cell growth in MDA-MB-231 human breast cancer cells. Of note, MDA-MB-231 human breast cancer cells have high levels of Bcl-2 and Bcl-xL proteins. Hence, (-)-gossypol is the active form of racemic gossypol. Accordingly, we have focused our investigation on (-)-gossypol in subsequent studies.

Because Bcl-2 and Bcl-xL proteins are central apoptosis regulators and potent cellular antagonists of apoptosis, we investigated whether gossypol and its enantiomers could trigger apoptosis in cancer cells with high levels of Bcl-2 protein and have selectivity to normal cells. The results for MDA-MB-231 cancer cells and normal WI-38 fibroblast cells treated with 20 μM of gossypol for 24 hours as detected by the Hoechst Dye assay are shown in **Figures 5A** and **5B**. These results demonstrated that gossypol induces apoptosis in MDA-MB-231 breast cancer cells with high levels of Bcl-2 and Bcl-xL, but not in normal fibroblast WI-38 cells with low levels of Bcl-2 and Bcl-xL expression.

To more quantitatively test the potency of gossypol in induction of apoptosis in MDA-MB-231 breast cancer cells, further tests using an Annexin-V flow cytometry (FACS) assay were performed (**Figure 5C**). It was observed that induction of apoptosis by gossypol is dose-dependent. At 5.0, 10 and 20.0 μM, gossypol caused 59%, 74% and 96% of the cells to undergo apoptosis, respectively.

Figure 4. Inhibition of cell growth by gossypol, (-)-gossypol and (+)-gossypol in human breast cancer cell line MDA-MB-231 cell line

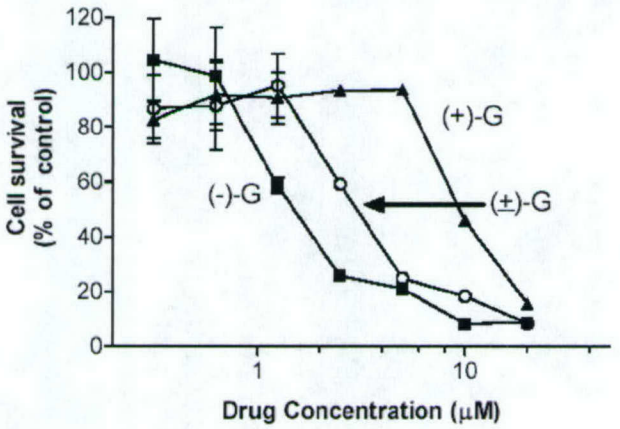
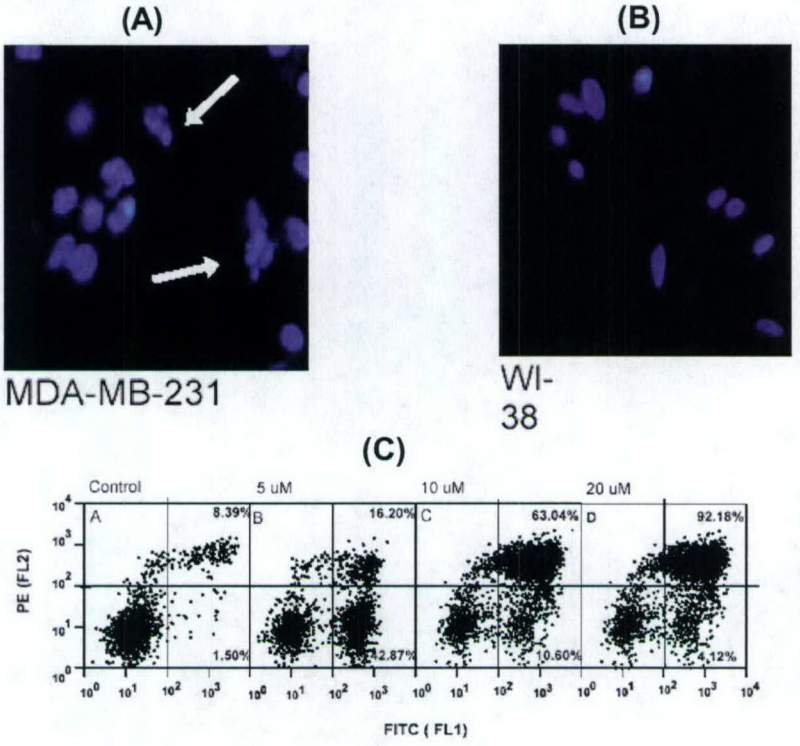


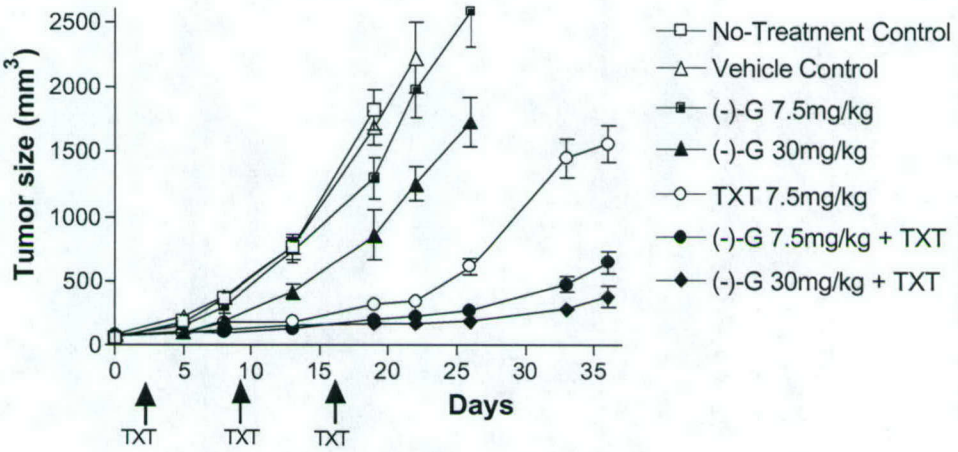
Figure 5. Induction of apoptosis in MDA-MB-231 cells and selectivity in normal fibroblast WI-38 cells.



Task 3. *In vivo* testing of 2-3 most promising lead compounds (**24-36 months**).

Based upon our *in vitro* data, (-)-gossypol potently inhibits cancer cell growth and induce apoptosis in cancer cells with high levels of Bcl-2 and Bcl-xL proteins. Importantly, (-)-gossypol has a good selectivity to normal cells. Hence, (-)-gossypol may represent a potent and highly promising small-molecule inhibitor of Bcl-2 and Bcl-xL and warrants further investigation for its therapeutic potential for the treatment of human breast cancer. We have therefore synthesized grams of quantity of (-)-gossypol and carried out *in vivo* studies to test its anti-tumor activity in animal model of human breast cancer using the MDA-MB-231 xenograft model in nude mice. The results are summarized in **Figure 6**. Each group of mice consisted of at least 5 mice and 10 tumors. As can be seen, (-)-gossypol has a dose-dependent, potent activity in inhibition of tumor growth. At 30 mg/kg (p.o. daily dose for 4 weeks), (-)-gossypol achieved more than 50% of tumor growth inhibition. Of note, (-)-gossypol is an orally available agent, a major advantage in the development of a novel anti-cancer drug.

Figure 6. *In vivo* antitumor activity of (-)-gossypol ((-)-G) in the MDA-MB-231 (2LMP) xenograft model in nude mice, alone or in combination with taxotere (TXT).



Our hypothesis predicted that a potent small-molecule inhibitor may achieve a greater anti-tumor activity when used in combination with chemotherapeutic agents. To test this hypothesis, we have tested the anti-tumor activity of (-)-gossypol in combination with a commonly used anti-cancer agent Taxotere (TXT). The results are shown in **Figure 6**.

As can be seen, although TXT (7.5 mg/kg, *i.v.* weekly for three weeks) has a good anti-tumor activity, the combination with either 7.5 mg/kg or 30 mg/kg of (-)-gossypol achieved a much greater anti-tumor activity than either agent alone. Overall, greater than 95% of tumor growth inhibition was achieved when (-)-gossypol was combined with TXT. The results are also highly statistically significant ($p < 0.001$).

Taken together, our data showed that (-)-gossypol achieves a good anti-tumor activity *in vivo* in inhibition of tumor growth when used as a single agent. Importantly (-)-gossypol achieves a much greater anti-tumor activity *in vivo* when used in combination with TXT. These data indicate that (-)-gossypol may have great therapeutic potential to be developed as a novel class of anti-cancer drug for the treatment of human breast cancer and other types of cancer, through, at least in part by inhibition of Bcl-2 and Bcl-xL proteins.

Task 4. Preparing scientific publications (**6-36 months**).

The following manuscripts have been accepted for publications or in preparation. The DOD grant support is acknowledged in these manuscripts.

1. Zaneta Nikolovska-Coleska, Dajun Yang, York Tomita[3], Peter P. Roller, Shaomeng Wang, Structure Based Discovery Of Gossypol as an Inhibitor Of Bcl-2 Family Proteins and Caracterization of Gossypol Enantiomers, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

2. Dajun Yang, Manchao Zhang, Jianyong Chen, Zaneta Nikolovska-Coleska, Liang Xu, Marc E. Lippman, York Tomita[3], Peter P. Roller, Shaomeng Wang, Molecular Mechanism and Pre-Clinical Testing Of (-)-Gossypol as a Potent Inhibitor of Bcl-2 and Bcl-xL for Apoptosis Targeted Anti-Cancer Therapy, manuscript in preparation and to be submitted to *Cancer Research.*

3. Christopher L. Oliver, Joshua A. Bauer, Mathew L. Ubell, Ajita Narayan, Kathleen M. O'Connell, Susan G. Fisher, Shaomeng Wang, Xihan Wu, Min Ji, Thomas E. Carey, and Carol R. Bradford, Small Molecule Inhibition of Bcl-xL and Bcl-2 as a Novel Therapeutic Strategy For Treatment of Head and Neck Squamous Cell Carcinoma, Clinical Cancer Research, (*in press*).

4. Ramzi M. Mohammad, Shaomeng Wang, Xihan Wu, Jianyong Chen, Amro Aboukameel, Ayad Al-Katib, Preclinical studies of (-)-Gossypol, a potent small molecule inhibitor of Bcl-2 and Bcl-xL, against diffuse large cell lymphoma (DLCL) xenograft model, Molecular Cancer Therapeutics, (*in press*).

5. Liang Xu, Dajun Yang, Shaomeng Wang, Wenhua Tang, Meilan Liu, Mary Davis, Jianyong Chen, James M. Rae, Theodore Lawrence and Marc E. Lippman, (-)-Gossypol, a Natural Polyphenol Inhibitor of Bcl-2/XL, Enhances Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer, Molecular Cancer Therapeutics, (**accepted with revisions**).

Key Research Accomplishments:

(1). We have discovered and characterized gossypol and its enantiomers as potent small-molecule inhibitors of Bcl-2 and Bcl-xL.

(2). We demonstrated that (-)-gossypol has potent activity in human breast cancer cells and other cancer cells with high levels of Bcl-2 and Bcl-xL proteins and show good selectivity in normal cells with low levels of Bcl-2/Bcl-xL proteins.

(3). We showed that (-)-gossypol achieves a significant anti-tumor activity in vivo in inhibition of tumor growth in the MDA-MB-231 xenograft model and has a much greater anti-tumor activity when used in combination with taxotere than either agent alone.

(4). Based upon our discovery, (-)-gossypol is currently being advanced into Phase I human clinical trials as a novel agent for the treatment of human breast cancer and other types of cancer in which Bcl-2/Bcl-xL proteins are highly overexpressed and traditional therapies have failed. If successful, our research will benefit thousands of cancer patients in the near future.

Reportable Outcome s:

1. Zaneta Nikolovska-Coleska, Dajun Yang, York Tomita[3], Peter P. Roller, Shaomeng Wang, Structure Based Discovery Of Gossypol as an Inhibitor Of Bcl-2 Family Proteins and Caracterization of Gossypol Enantiomers, (manuscript in preparation and to be submitted to *Journal of Medicinal Chemistry*).

2. Dajun Yang, Manchao Zhang, Jianyong Chen, Zaneta Nikolovska-Coleska, Liang Xu, Marc E. Lippman, York Tomita[3], Peter P. Roller, Shaomeng Wang, Molecular Mechanism and Pre-Clinical Testing Of (-)-Gossypol as a Potent Inhibitor of Bcl-2 and Bcl-xL for Apoptosis Targeted Anti-Cancer Therapy, manuscript in preparation and to be submitted to *Cancer Research.*

3. Christopher L. Oliver, Joshua A. Bauer, Mathew L. Ubell, Ajita Narayan, Kathleen M. O'Connell, Susan G. Fisher, Shaomeng Wang, Xihan Wu, Min Ji, Thomas E. Carey, and Carol R. Bradford, Small Molecule Inhibition of Bcl-xL and Bcl-2 as a Novel Therapeutic Strategy For Treatment of Head and Neck Squamous Cell Carcinoma, Clinical Cancer Research, (*in press*).

4. Ramzi M. Mohammad, Shaomeng Wang, Xihan Wu, Jianyong Chen, Amro Aboukameel, Ayad Al-Katib, Preclinical studies of (-)-Gossypol, a potent small molecule inhibitor of Bcl-2 and Bcl-xL, against diffuse large cell lymphoma (DLCL) xenograft model, Molecular Cancer Therapeutics, (*in press*).

5. Liang Xu, Dajun Yang, Shaomeng Wang, Wenhua Tang, Meilan Liu, Mary Davis, Jianyong Chen, James M. Rae, Theodore Lawrence and Marc E. Lippman, (-)-Gossypol, a Natural Polyphenol Inhibitor of Bcl-2/XL, Enhances Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer, Molecular Cancer Therapeutics, (**accepted with revisions**).

6. An invention has been filed with University of Michigan on gossypol and its analogues as a new class of anti-cancer agents.

Conclusions:

Although this Award was only received in July, 2003 due to the transfer of the grant from Georgetown University to University of Michigan, we have well on our way to accomplish all the major goals and tasks we proposed in our original proposal. More than 10 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2 have been successfully identified. Three manuscripts are now in press or two additional manuscripts will be submitted soon. An invention disclosure has been filed with University of Michigan. In summary, this has become a highly productive project. Among those small-molecule inhibitors we have discovered, (-)-gossypol, is a potent Bcl-2/Bcl-xL inhibitor and represents a highly promising anti-cancer agent. Based upon our results, a Phase I clinical trial is currently being planned to evaluate (-)-gossypol as a new anti-cancer agent for the treatment of human breast and other types of cancer.